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Recruitment of Ectodermal Attachment Cells via an EGFR-Dependent Mechanism during the Organogenesis of *Drosophila* Proprioceptors

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Summary

***Drosophila* proprioceptors (chordotonal organs) are structured as a linear array of four lineage-related cells: a neuron, a glial cell, and two accessory cells, called cap and ligament, between which the neuron is stretched. To function properly as stretch receptors, chordotonal organs must be stably anchored at both edges. The cap cells are anchored to the cuticle through specialized lineage-related attachment cells. However, the mechanism by which the ligament cells at the other edge of the organ attach is not known. Here, we report the identification of specialized attachment cells that anchor the ligament cells of pentascolopidial chordotonal organs (lch5) to the cuticle. The ligament attachment cells are recruited by the approaching ligament cells upon reaching their attachment site, through an EGFR-dependent mechanism. Molecular characterization of lch5 attachment cells demonstrated that they share significant properties with *Drosophila* tendon cells and with mammalian proprioceptive organs.**

Introduction

Proprioceptors are sensory organs that detect and relay information concerning movements and position of the body. In the peripheral nervous system (PNS) of *Drosophila* and other insects, the subepidermal chordotonal (ch) organs function as stretch receptors that provide proprioception. The ch organ is structured as a linear array of four different cells, all of which are the progeny of a single chordotonal organ precursor (COP). Each organ (or scolopidium) contains a bipolar neuron whose dendrite is ensheathed by a scolopale cell and two accessory cells between which the neuron is stretched: the cap cell at the dendritic side and the ligament cell at the axonal side (Bodmer et al., 1989). A critical step in the development of a ch organ is the attachment of both its edges to fixed positions. The anchoring allows the organ to withstand tension and function as a stretch receptor. Several ways in which the cap cell can attach to the cuticle have been described, based on morphological studies of different types of ch organs (for a review, see Field and Matheson, 1998). However, very little is known about the developmental program that regulates the attachment process. Thus, the mechanisms that direct the cap and ligament cells to anchor at precise positions and the mechanisms that control cell fate determination and differentiation of the cells through which they attach are yet to be elucidated.

To gain a better insight into the process of ch attachment cell development, we focused on the attachment cells of the pentascolopidial organs. These organs, also known as the five lateral ch (lch5) organs, are located in the lateral region of each of abdominal segments A1 through A7 of the *Drosophila* embryo (Ghysen et al., 1986). The formation of lch5 begins with the appearance of three COPs that express high levels of Atonal (Ato) (Jarman et al., 1993). Ato triggers the expression of Rhomboid (Rho) in the precursors, leading to the activation of the epidermal growth factor receptor (EGFR) pathway in neighboring cells and resulting in the recruitment of two additional precursors (Okabe and Okano, 1997; zur Lage et al., 1997). The five COPs then go through several asymmetric cell divisions to form the five ch organs comprising each pentascolopidial organ. The cap cells of these organs are attached on their dorsal side to two ectodermally located cells, which originate from the ch lineage as well and are thought to anchor the cap cells to the cuticle (Matthews et al., 1990; Brewster and Bodmer, 1995).

The precursors of lch5 are born in the dorsal region of the embryo, yet the mature organs are located in the lateral PNS cluster (Bier et al., 1990; Salzberg et al., 1994). Based on the positions of the various lch5 cells during different developmental stages, we have recently proposed a two-step model for the process of lateral localization of lch5: the organs first rotate to assume a correct orientation and then stretch ventrally to reach their final position (Inbal et al., 2003). The ligament cells seem to be the leading cells in the stretching process, at the end of which they must form a stable attachment to allow the organ to function as a stretch receptor. The mechanisms controlling the migration and targeting of the ligament cells toward their attachment sites have not been characterized. In addition, it is not known whether the ligament cells, similarly to the cap cells, attach to the cuticle through specialized cells and, if so, what the identity of these cells is and what regulates their determination and differentiation.

In this work, we show that the ligament cells of each pentascolopidial organ are anchored to the ectoderm by a specialized attachment cell. Whereas the cap cells attach to the ectoderm via lineage-related cells, our data show that the ligament cells recruit their attachment cells from the ectoderm and induce their specialization. This recruitment takes place only when the ligament cells reach their final position and is achieved by an EGFR pathway-dependent mechanism. Molecular characterization of the attachment cells at both edges of lch5

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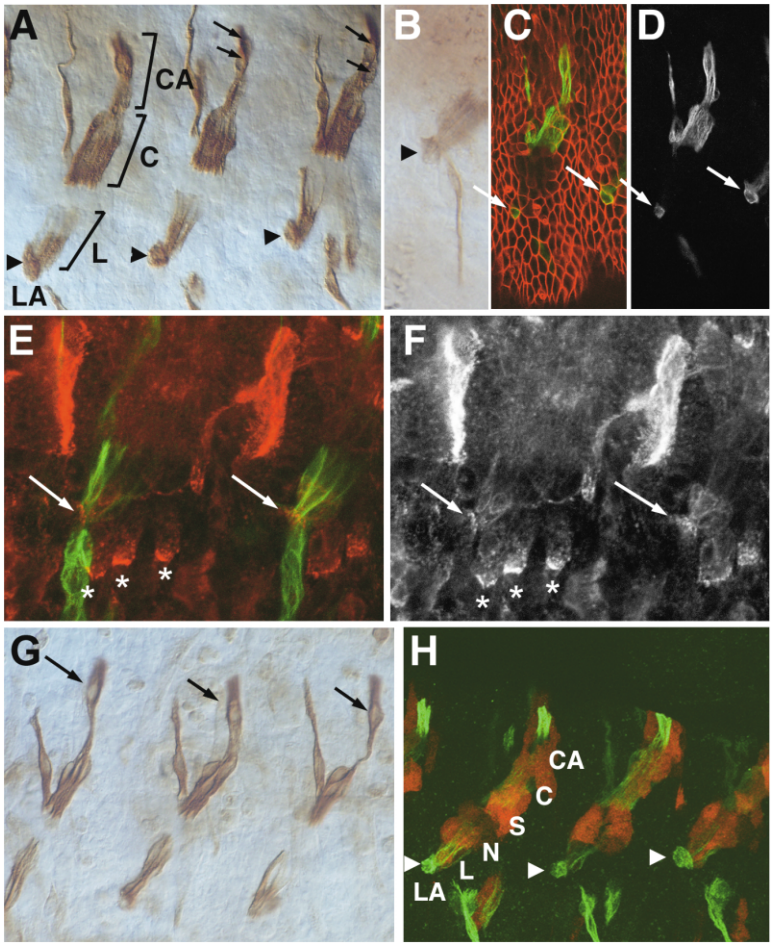

Figure 1. Attachment Cells of ch Organs

(A and B) Abdominal segments of a wild-type embryo (A) and a first instar larva (B) stained with anti-α85E, revealing ligament attachment (LA) cells (arrowheads) and cap attachment (CA) cells (arrows).

(C and D) Double staining with anti-Fas3 (red) and anti-α85E (green in [C], white in [D]) demonstrates the localization of the LA cells (arrows) in the epidermal layer.

(E and F) Double staining with anti-βPS-Integrin (red) and anti-α85E (green in [E], white in [F]). High concentration of βPS-Integrin is evident in the site of attachment between ligament and LA cells (arrows). The asterisks mark high concentration of βPS-Integrin in muscle attachment sites.

(G) A *spi*^{OE92} mutant embryo. Only a single CA cell (arrow) is observed in each of the lateral ch organs, which are comprised of only three instead of five scolopidia.

(H) An *ato-Gal4/UAS-lacZ* embryo stained with anti-α85E (green) and anti-β-Gal (red). All cells of the lch5 organ except for the LA cell (arrowheads) express β-Gal.

The following abbreviations are used in all figures: LA, ligament attachment cells; L, ligament cells; N, neurons; S, sheath cells; C, cap cells; CA, cap attachment cells. Here and in all following panels, abdominal segments of stage 16–17 embryos are shown, unless mentioned otherwise. Anterior is to the left and dorsal is up.

organs revealed that they share significant molecular properties with tendon cells, which anchor muscles to the cuticle.

Results and Discussion

Attachment Cells of lch5 Organs

In an attempt to characterize the origin and fate of ch attachment cells, we examined the distribution of α85E-tubulin (α85E-tub) in ch organs. This minor α-tub variant is known to be expressed in the cap cells and the adjacent attachment cells, as well as in the ligament cells of lch5 organs (Matthews et al., 1990). Close inspection of the distribution of this protein in mature embryos and first instar larvae revealed another α85E-tub-expressing cell in close proximity to the ventral edge of the ligament cells (Figures 1A and 1B). Rarely, two such cells were observed. These large cells appeared to be good candidates to function in the attachment of ligament cells. Indeed, further analysis demonstrated that these cells are localized within the epidermal layer (Figures 1C and 1D) and are connected to the ventral edges of the ligament cells via Integrin-mediated adhesion, as suggested by the high concentration of the Integrin βPS subunit in the contact site between these two cell types (Figures 1E and 1F). In addition, these cells possess many features that are typical of other types of attachment cells (see below). To avoid confusion, we hereafter refer to the attachment cells that anchor the cap cells as CA (cap attachment) cells and to the attachment cells that anchor the ligament cells as LA (ligament attachment) cells.

Lineage-tracing experiments have shown that the CA cells originate from the ch organ lineage (Brewster and Bodmer, 1995). This observation predicts the formation of five CA cells in each of the lateral pentascolopidial organs. However, only two CA cells can be identified in each of these organs (Matthews et al., 1990; Brewster and Bodmer, 1995; Figure 1A). It was previously suggested that the other three cells either degenerate or migrate away from the cluster (Brewster and Bodmer, 1996). In order to identify which of the five ch organs generate a CA cell, we examined *spitz* (*spi*) and *rhomboid* (*rho*) mutant embryos in which only the first three ch organs are formed (Bier et al., 1990; Okabe and Okano, 1997; zur-Lage et al., 1997; Rutledge et al., 1992). In both types of mutants, only one CA cell was detected in each pentascolopidial organ (Figure 1G and data not shown). This observation suggests that one of the CA cells originates from one of the first three ch organs, whereas the second cell is formed by one of the two organs that are recruited later.

The LA cell was not detected in the lineage-tracing experiments mentioned above, suggesting that this cell

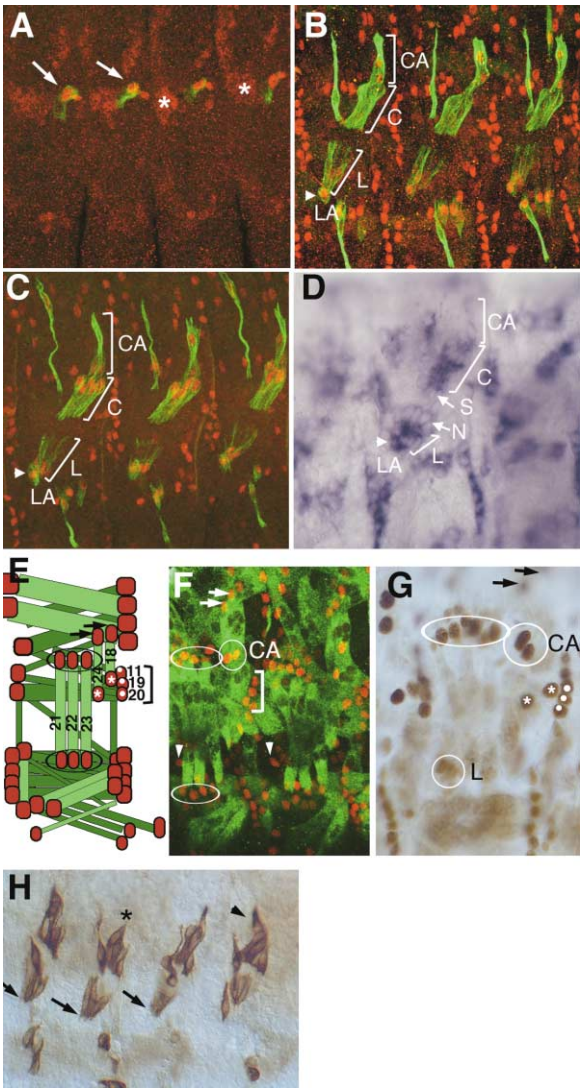

Figure 2. ch Attachment Cells Express Tendon Cell-Typical Markers and Require Sr Activity for Their Development

(A and B) Wild-type embryos stained with anti-Sr (red) and anti-α85E (green). (A) A stage 13 embryo. Strong Sr expression is observed in the CA cells (arrows). Low levels of Sr are evident in clusters of tendon precursor cells (asterisks). (B) At stage 16, Sr is expressed in the CA cells, LA cells (arrowheads), and ligament cells.

(C) A wild-type embryo stained with anti-Dei (red) and anti-α85E (green). Dei expression is observed in CA cells, cap cells, LA cells (arrowheads), and ligament cells.

(D) In situ hybridization with a *β1-tub* probe to a wild-type embryo, showing *β1-tub* transcripts in the LA cell (arrowhead), ligament cells, cap cells, and CA cells (not in focus). Very weak expression is seen also in the neurons.

(E) A schematic view of body wall muscles (green) and tendon cells (red). Tendon cells of muscles 21 to 23 are circled, and the dorsal tendon cells of muscles 24 and 18 are indicated by arrows. The ventral tendon cells of these muscles are indicated by asterisks. The white dots label the tendon cells that attach muscles 11, 19, and 20.

(F and G) *sr-lacZ* embryos stained with anti-β-Gal (G) or anti-β-Gal (red) and anti-Myosin heavy chain (green) (F) to reveal the relative positions of ch and muscle attachment cells. Tendon cells of the relevant muscles are marked as in (E). The CA cells and ligament cells are circled. The arrowheads in (F) point to LA cells. Note that none of the muscles attach in the region of the LA cells and that the CA cells do not colocalize with attachment sites of any of the muscles in that region.

(H) A sr^{155}*/Df(3R)DG4* embryo stained with anti-α85E. CA cells (arrowhead) appear to be abnormal and are missing from one of the lch5 organs (asterisk). Arrows point to the edges of ligament cells, where LA cells are normally found. lch5 organs are not fully stretched (compare with Figure 1A).

is not related to the ch lineage. To verify this conclusion, we stained *ato-Gal4/UAS-lacZ* embryos, which express β-Galactosidase (β-Gal) in all cells of the ch lineage, with anti-β-Gal and anti-α85E-tub. Indeed, the LA cell was the only α85E-tub-positive cell that did not express β-Gal (Figure 1H), confirming that it did not originate from the ch lineage.

The function of ch organs as stretch receptors requires the stable attachment of both their edges to fixed positions. The identification of LA cells provides an answer to the question of how lch5 organs attach through their ligament cells. However, this does not seem to be the case for all ch organs in the embryonic PNS. For example, the ventral ch organs A and B (vchA and vchB) have no apparent ligament cells and LA cells (data not shown). Thus, different subtypes of ch organs exist, which differ in their structure as well as attachment mechanisms.

lch5 Attachment Cells Share Some Molecular Properties with Muscle Attachment Cells

To learn more about the structural and molecular features of ch attachment cells, we tested whether these cells share any molecular properties with tendon cells, which attach muscles to the cuticle. Tendon cells have been extensively studied, and several genes that are involved in their differentiation were identified (reviewed in Volk, 1999). Since both tendon and ch attachment cells are designed to resist mechanical strain, we examined whether the ch attachment cells express tendon cell-typical markers. The formation of tendon cells requires the expression of Stripe (Sr), an early growth response (EGR)-like transcription factor (Lee et al., 1995; Frommer et al., 1996). Sr induces the expression of an array of tendon cell-specific proteins, which are required for tendon cell differentiation (Volk, 1999). Double labeling wild-type embryos for α85E-tub and Sr revealed that Sr is expressed in ch organs in the CA, LA, and ligament cells. Sr expression was first detected in the CA cells at stage 13 (Figure 2A). CA cells are the first to express Sr in the embryo and seem to express the highest levels of Sr throughout embryonic development. The ligament cells expressed lower levels of Sr from late stage 14 onward, and the LA cells expressed Sr in stage 16 or older embryos (Figure 2B).

Two other genes that are implicated in tendon cell terminal differentiation are *delilah* (*dei*), which encodes a bHLH transcription factor (Armand et al., 1994), and *β1-tubulin* (*β1-tub*; Buttgereit et al., 1991). Expression of both genes was reported in ch organs; however, their exact distribution within these organs has not been described. Double labeling wild-type embryos for α85E-tub and Dei revealed expression of Dei in the CA and LA cells and in the cap and ligament cells (Figure 2C).

In situ hybridization revealed that β*1-tub* is expressed similarly to Dei. Very low levels of β*1-tub* transcripts were observed in addition in lch5 neurons (Figure 2D). Work done in tendon cells has shown that the expression of Dei and β*1-tub* is induced by Sr in a cell-autonomous manner (Frommer et al., 1996; Becker et al., 1997; Vorbrüggen and Jäckle, 1997). The fact that in lch5 organs the expression of Dei and β*1-tub* is not limited to Sr-expressing cells suggests that additional mechanisms control the expression of these genes. Thus, the differential distribution of α85E-tub, Sr, Dei, and β1-tub in the cells of lch5 organs adds a new dimension of complexity to these organs and raises new questions regarding the regulation of gene expression, cell fate determination, and differentiation in each cell type.

Despite the similarities between tendon and ch attachment cells, muscles and ch organs do not share the same attachment sites, and the CA and LA cells serve for the anchoring of lch5 organs only (Figures 2E–2G). One prominent difference between CA, LA, and tendon cells is the expression of the α85E-tub protein in ch attachment cells but not in tendon cells. This suggests that α85E-tub has a unique function that is required in ch organs. It has been suggested that this isoform of α-tubulin, which is expressed specifically in ch organ accessory cells, developing muscles, and testis cyst cells, is likely to function in cells that must elongate extensively (Matthews et al., 1990). Thus, the contribution of the α85E-tub to the organization of the microtubule cytoskeleton in the ch organ accessory cells is likely to affect the elasticity of the cells and their ability to withstand tension.

Sr Is Required for CA Cell Differentiation and LA Cell Formation

Sr functions at the top of the hierarchy to direct tendon cell differentiation. In the absence of Sr, tendon cells do not develop, and the muscles fail to attach to the ectoderm (Frommer et al., 1996). To test the role of Sr in the formation of lch5 attachment cells, we examined how *sr* loss of function affects these cells. Staining *sr* mutant embryos with anti-α85E-tub revealed a loss of LA cells in the absence of Sr. The CA cells were only occasionally missing; however, their morphology appeared to be abnormal (Figure 2H). The lch5 organs were not properly stretched and appeared to be somewhat collapsed (Figure 2H), possibly as a result of their failure to form stable attachments to the ectoderm. Thus, Sr is required for the generation of functional lch5 organs by playing a role in the formation of LA cells and in the differentiation of CA cells.

It is not surprising that the two types of lch5 attachment cells are affected differently by the loss of Sr function. The earliest expression of Sr in the CA cells is observed in stage 13 embryos, after all cells of lch5 organs have already formed. Thus, Sr is not expressed early enough to affect primary decisions of cell fate in the lch5 lineage, but it may represent the earliest marker of the fate acquired by CA cells. As for the LA cells, their identity is defined very late in embryonic development, and Sr expression seems to be the earliest sign of their existence. Thus, Sr is likely to play a role in their induction as well as their differentiation into attachment cells.

Sr is a member of the EGR family of transcription factors. In mammals, EGR proteins are involved in multiple developmental processes (reviewed in O'Donovan et al., 1999). Egr3, which shows a significant sequence similarity to Sr, is expressed in differentiating muscle spindles, a subgroup of proprioceptors. In Egr3 null mice, these proprioceptors are missing as a result of their failure to differentiate (Tourtellotte and Milbrandt, 1998; Tourtellotte et al., 2001). Thus, an intriguing molecular parallelism might exist between the formation and differentiation of *Drosophila* and mammalian proprioceptive organs, despite the significant differences in their structure.

LA Cells Are Induced by the lch5 Ligament Cells

The fact that the LA cells do not belong to the ch lineage raises the question of what triggers their formation. We have recently shown that lch5 organs are initially formed with their ligament cells in a relatively dorsal position; subsequently, these cells descend until they reach their final position in the lateral cluster (Inbal et al., 2003). Thus, the late appearance of the LA cells presents two possibilities with regard to their induction: these cells could form at a late embryonic stage independently of the ligament cells, or they could be recruited by the approaching ligament cells. To find which of these possibilities is correct, we examined embryos in which the ligament cells were ablated, relatively late in development, by expressing in them the apoptosis-inducing gene *rpr*, or mutant embryos in which ligament cells do not form due to mutation in the *gcm* or *repo* genes (Jones et al., 1995; Hosoya et al., 1995; Halter et al., 1995). In the absence of ligament cells, the LA cells could not be detected (Figures 3C and 3D and data not shown), suggesting that their formation depends on the presence of ligament cells. When the ligament and LA cells are missing, the lch5 organs are not fully stretched, and the cap cells appear shorter than normal (compare Figures 3A and 3B to Figures 3C and 3D). However, different types of connections between the lch5 cells and their environment (e.g., the fasciculation of the lch5 axons with the intersegmental nerve) prevent a complete collapse of these organs in the absence of their ventral anchor.

To find whether the presence of ligament cells is sufficient to induce the formation of LA cells regardless of their position, we examined embryos in which the ligament cells were abnormally localized. Mutations in *abdominal-A* (*abd-A*), *homothorax* (*hth*), and *ventral veinless* (*vvl*) result in frequent dorsal localization of lch5 organs (Heuer and Kaufman, 1992; Salzberg et al., 1994; Kurant et al., 1998; Inbal et al., 2003). lch5 organs that fail to localize to their correct position in these mutants do not have LA cells (Figure 3E and data not shown). However, since the protein products of *abd-A*, *hth*, and *vvl* are normally expressed in the ectoderm, it is possible that, in their absence from the ectoderm of mutant embryos, LA cells cannot develop, regardless of the positioning of ligament cells. To assess specifically the influence of ligament cell positioning, we utilized an inducible Hth antimorph (En-Hth$^{1\text{-}430}$; Inbal et al., 2001) that can phenocopy *hth* loss of function. Expression of this antimorph in ch organs under the regulation of *ato-Gal4*

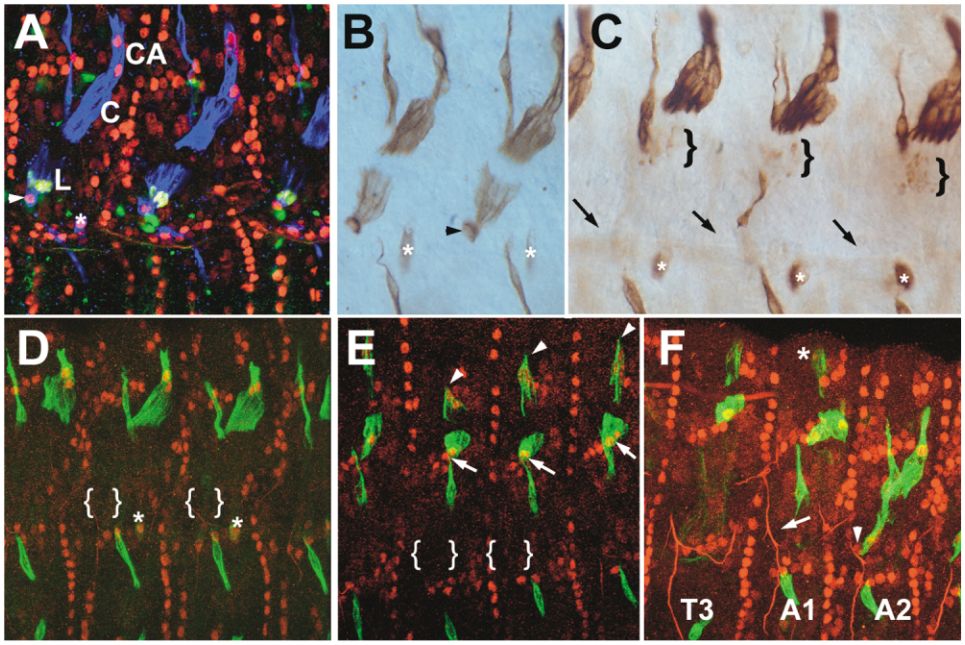

Figure 3. Differentiation of LA Cells Depends on the Presence of Ligament Cells in the Correct Position

(A) A normal stage 16 embryo stained with anti-α85E (blue), anti-Sr (red), and anti-Repo (green). Repo expression is evident in the ligament cells (L) but not in the LA cells (arrowhead).

(B and C) Abdominal segments of stage 16 embryos collected from a *repo-Gal4/TM6* × *UAS-rpr* cross and stained with anti-α85E. (B) An embryo that did not inherit the *repo-Gal4* transgene presents normal ligament and LA cells (arrowhead). (C) The expression of *rpr* in the ligament cells led to their programmed death (debris of the dying cells are marked with parentheses). The loss of ligament cells led to consequent loss of LA cells (arrows). The asterisks in (A)–(D) mark α85E-expressing cells of unknown nature that were not affected in these experiments.

(D) A *gcm* mutant embryo. Ligament cells and LA cells are missing. White parentheses mark the region in which LA cells are normally found.

(E) An *abd-A* mutant embryo. The orientation of lch5 organs is reversed: ligament cells are more dorsal than the CA and cap cells (arrows). The arrowheads point to the edges of ligament cells. No LA cells can be observed near the edge of the aberrantly positioned ligament cells or where they are normally found (white parentheses).

(F) An *ato-Gal4/UAS-En-Hth*$^{1-430}$ embryo. The lch5 organs of abdominal segment A1 display abnormal position and orientation that resemble those of the dorsal thoracic ch organs (seen in T3). The lch5 organs in A2 are localized properly, and their ligament cells are in the correct position. A single Sr-expressing cell that corresponds in position to the LA cell is observed in this segment (arrowhead), whereas in A1 there are no Sr-expressing cells in this region (arrow) or near the edge of the aberrantly positioned ligament cells (asterisk).

results in a high percentage of abnormally oriented lch5 organs (A.I. and A.S., unpublished data). Except for their abnormal positioning, lch5 organs in these embryos appear to be fully differentiated, as judged by their ability to express typical markers, such as Repo, α85E-tub, and Sr (Figure 3F and data not shown). In *ato-Gal4/UAS-En-Hth*$^{1-430}$ embryos, no LA cells could be observed in abdominal segments that exhibited abnormally oriented lch5 organs (Figure 3F). Altogether, these data suggest that lch5 ligament cells recruit their attachment cells and that this process is restricted spatially, perhaps due to competence of cells in the attachment site region.

The recruitment of LA cells by ligament cells resembles the recruitment of tendon cells by myotubes. In the case of tendon cells, the leading edges of myotubes approach preexisting clusters of Sr-expressing cells, and upon reaching their target they induce the terminal differentiation of a single tendon cell. The expression of Sr in the tendon precursor clusters also serves to attract the myotubes (Becker et al., 1997; Vorbrüggen and Jäckle, 1997). In the case of ligament cells, however, we could not detect clear expression of Sr in the prospective site of their attachment prior to the appearance of the LA cell (data not shown), which takes place only when the ligament cells are in their final position. Thus, despite the high similarity between the two processes, differences seem to exist in the mechanisms that guide ligament cells and myotubes to their attachment sites.

Formation of LA Cells Requires EGFR Pathway Activation

Tendon cells are induced by the approaching myotubes, which secrete the EGFR ligand Vein and activate the EGFR pathway in the tendon precursor cells that they contact. This activation results in the expression of tendon cell-typical markers and terminal differentiation of tendon cells (Yarnitzky et al., 1997). Since the approaching ligament cells seem to induce the LA cells that share many properties with tendon cells, we tested whether the EGFR pathway plays a role in the process of LA cell induction. We first tested for activation of the EGFR pathway within the developing LA cells by costaining wild-type embryos for the activated form of MAP kinase (dp-ERK; Gabay et al., 1997) and for α85E-tub. In stage 16 embryos, low levels of dp-ERK could be detected in the LA cells but not in any of the other

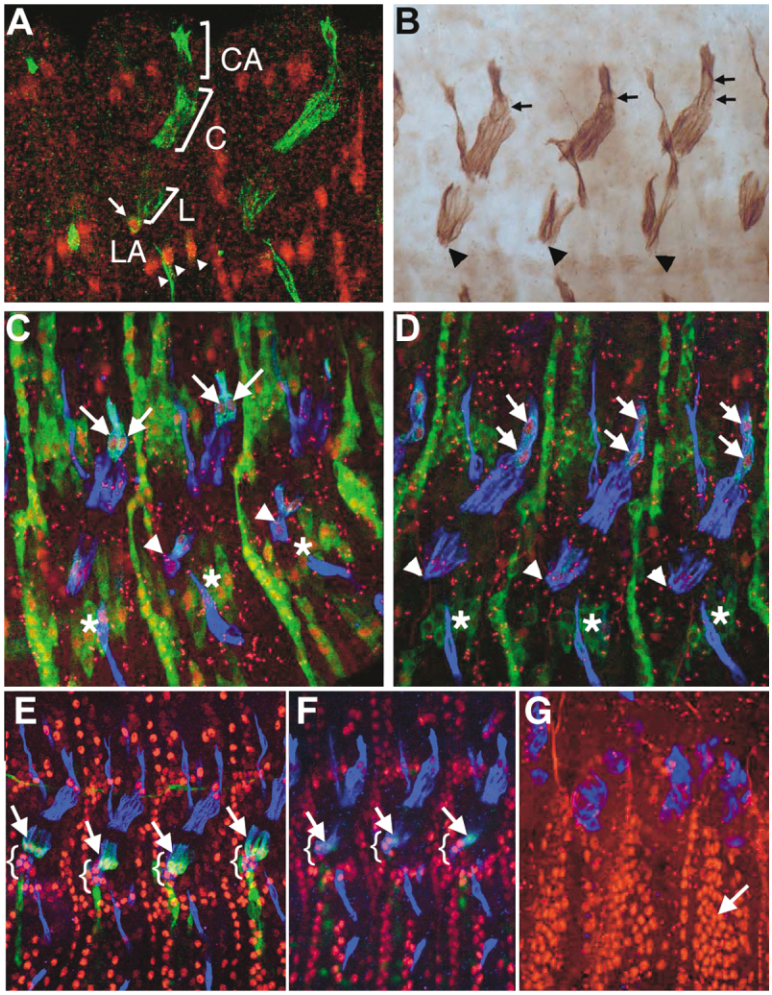

Figure 4. EGFR Pathway Activity Is Required for LA Cell Formation

(A) A wild-type embryo stained with anti-α85E (green) and anti-dp-ERK (red) to detect EGFR pathway activation. dp-ERK is observed in the LA cell (arrow) and in tendon cells (arrowheads).

(B) A *69B-Gal4/UAS-DN-DER* embryo stained with anti-α85E. LA cells are missing from their normal positions at the edge of ligament cells (arrowheads; compare to Figure 1A), whereas CA cells are present (arrows).

(C and D) Embryos carrying *UAS-GFP* and *sr-Gal4* transgenes, stained with anti-Sr (red) and anti-α85E (blue). GFP expression (green) marks Sr-expressing cells, including CA cells (arrows) and tendon cells (for example, ventral attachment cells of muscles 21 through 23 [asterisks]). The embryo in (D) carries in addition a *UAS-DN-DER* transgene. Note the absence of LA cells (arrowheads) and the reduction in the level of Sr expression in the tendon cells but not CA cells of this embryo (compare [C] and [D]).

(E and F) A *UAS-GFP/UAS-sSpi; repo-Gal4* embryo (E) and a *UAS-GFP/UAS-vein; repo-Gal4* embryo (F). Repo-expressing cells, including ligament cells (arrows), are marked by GFP expression (green). Supernumerary LA cells, which express Sr (red) and α85E (blue), are marked in parentheses (compare to Figure 2B).

(G) A *69B-Gal4/UAS-sSpi* embryo. The arrow points at some of the multiple ectopic Sr-expressing cells observed in these embryos. These cells do not express α85E.

lch5 cells (data not shown). Higher levels of dp-ERK were detected in the LA cells of stage 17 embryos (Figure 4A). These observations demonstrate that the MAP-kinase pathway is activated in the LA cells at the time of their formation. To establish whether this pathway is necessary for the induction of these cells and whether it is mediated through EGFR activation, we blocked specifically the EGFR pathway by expressing a dominant-negative form of the receptor (DN-DER; O'Keefe et al., 1997). The DN-DER was expressed throughout the ectoderm using the *69B-Gal4* driver or, in all of the Sr-expressing cells, including the LA cells, using a *sr-Gal4* driver. In both cases, the expression of DN-DER abolished the formation of LA cells (Figures 4B and 4D), indicating that activation of the EGFR pathway is necessary for LA cell development. To establish whether activation of the pathway plays a permissive or an instructive role in the formation of LA cells, we tested whether higher levels of EGFR activation can lead to the formation of supernumerary LA cells. To elevate the level of EGFR activation locally, we expressed the EGFR ligand Vein or a secreted form of the ligand Spitz (sSpi) in the ligament cells under the regulation of *repo-Gal4*. This excessive activation resulted in the formation of increased numbers of LA cells (Figures 4E and 4F), indicating that the EGFR pathway plays an instructive role in the induction of lch5 LA cells. Expression of sSpi throughout the ectoderm led to the induction of multiple ectopic Sr-expressing cells; however, these cells did not express the α85E-tub protein (Figure 4G), suggesting that EGFR pathway activity is required but not sufficient to determine the identity of an LA cell.

While EGFR pathway activity is clearly required for the generation of LA cells, CA cells did not seem to be affected significantly by localized blocking of EGFR signaling. Moreover, CA cells appear to be almost the only cells that continue to express high levels of Sr when the EGFR pathway is blocked (Figure 4D), suggesting that Sr expression in these cells is controlled by a different mechanism than in LA and tendon cells.

The Neuregulin-like EGFR Ligand Vein Is Required for the Induction of LA Cells

To identify which ligand activates the EGFR pathway in LA cell induction, we examined how the loss of Vein or Spi activity affects the formation of these cells. Since the secretion of an active form of Spi requires the cleavage of an inactive precursor by Rho (Sturtevant et al., 1993; Golembo et al., 1996; Urban et al., 2001), we also examined the effects of Rho loss of function on the development of LA cells.

In embryos hemizygous for the hypomorphic allele $vein^{P1749}$, LA cells could not be detected (Figure 5A). This observation suggests that Vein activity is required for

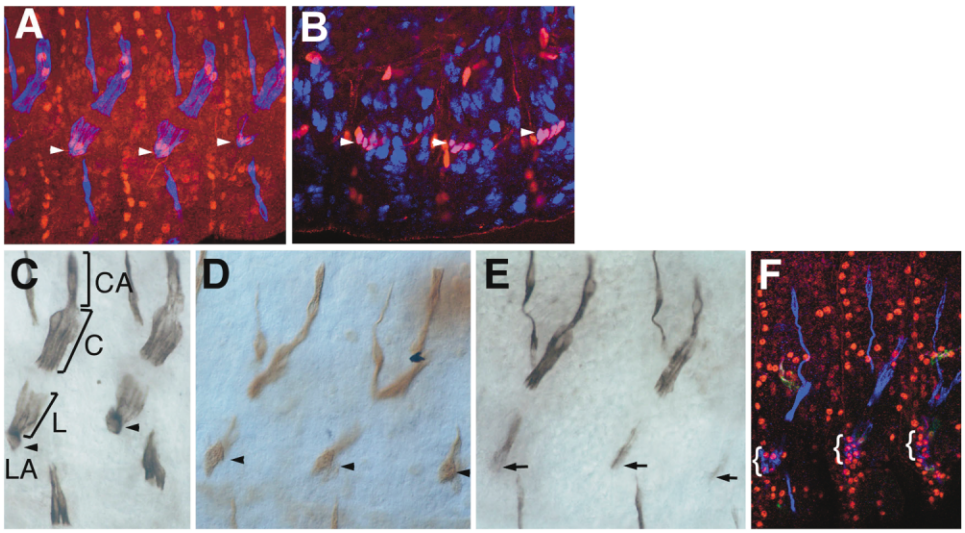

Figure 5. Vein Is Required for the Induction of LA Cells by Ligament Cells

(A) A *vein*^{P1749}*/Df(3L)v65c* embryo stained with anti-α85E (blue) and anti-Sr (red). LA cells are missing from their normal positions (arrowheads). Mutant embryos were identified based on the reduced level of Sr expression in tendon cells.

(B) A *vein*^{P1749} embryo costained with anti-β-Gal (blue) and anti-Repo (red). Arrowheads point to the β-Gal/Repo colocalization in the ligament cells.

(C–E) Anti-α85E staining of *rho*$^{\Delta 38}$ heterozygous (C), *spi*^{OE92} homozygous (D), and *rho*$^{\Delta 38}$ homozygous embryos (E). Homozygous *spi*^{OE92} or *rho*$^{\Delta 38}$ embryos were identified based on the presence of three instead of five scolopidia in each lateral ch organ. Arrowheads in (C) and (D) point to LA cells. Arrows in (E) point to the ventral edge of the ligament cells. Note the reduced expression of α85E in the ligament cells and the lack of LA cells in this embryo.

(F) A *rho*$^{\Delta 38}$ homozygous embryo in which the expression of sSpi was driven in ligament cells under the regulation of *repo-Gal4*. Note the excess of LA cells (parentheses) that express the combination of Sr (red) and α85E (blue).

the formation of LA cells. To verify this conclusion, we examined whether *vein* is expressed in the ligament cells at the time of LA cell induction. We performed in situ hybridization with a *vein*-specific probe and examined the *lacZ* expression pattern in embryos carrying the *vein*^{P1749} chromosome. This chromosome carries a P element insertion in the first noncoding exon of *vein*, which induces *lacZ* expression in a pattern similar to that of *vein* mRNA (Yarnitzky et al., 1997). Low levels of *vein* transcripts were present in the region of the ligament cells in stage 16 embryos (data not shown), but the expressing cells could not be identified unambiguously. Examining the expression pattern of β-Gal in *vein*^{P1749} embryos revealed that it is expressed in the ligament cells of stage 16 embryos (Figure 5B). Altogether, these data suggest that Vein is expressed in the lch5 ligament cells in late stages of embryogenesis and that Vein is the major ligand responsible for EGFR activation in the prospective LA cells. The ability of Vein to induce supernumerary LA cells when overexpressed in the ligament cells is consistent with this conclusion.

The role of Vein in the induction of LA cells extends the molecular similarity between the development of lch5 organs and mammalian proprioceptors. It was shown that Neuregulin1, a Vein homolog, is secreted from proprioceptive afferent nerve endings and is required for the expression of Egr3 and differentiation of muscle spindles in the mouse (Hippenmeyer et al., 2002).

To test for a possible involvement of Spi and Rho in the induction of LA cells, we examined *spi* and *rho* mutant embryos. In embryos homozygous for the strong hypomorphic allele *spi*^{OE92}, LA cells could be identified in 49% of the abdominal segments when visualized with anti-α85E-tub, as compared to 74% in heterozygous or wild-type sibling embryos (n = 49 and 91 segments, respectively). The LA cells often exhibited abnormal morphology and expressed relatively low levels of the α85E-tub protein (Figure 5D). In embryos homozygous for the null allele *rho*$^{\Delta 38}$, we could identify LA cells in only 20% of the segments (n = 49; Figure 5E), as compared to 54% of the segments in sibling embryos (n = 154; Figure 5C). Similarly to the *spi*^{OE92} mutant embryos, the LA cells in these embryos often appeared to be abnormal (data not shown).

The frequent loss of LA cells in *rho* and *spi* mutant embryos could indicate that these genes also contribute to the activation of the EGFR pathway and the induction of LA cells. To establish whether Rho plays a direct role in the induction of LA cells, we looked for its expression in ligament cells of stage 15–16 embryos. In situ hybridization with an antisense *rho* probe did not detect *rho* mRNA in ligament cells (data not shown). Similar results were obtained by staining whole embryos with anti-Rho antibodies. Furthermore, we could not detect β-Gal expression in the ligament cells of stage 15–16 embryos carrying the X81 *rho-lacZ* reporter construct (Freeman et al., 1992), with the exception of the most anterior scolopidium. This scolopidium originates from the C1 precursor, which expresses the highest levels of *rho* in early stages of lch5 development (Freeman et al., 1992; zur Lage et al., 1997). Due to the stability of the β-Gal protein, it can be detected in the progeny of C1 throughout embryonic development and is therefore not indicative in this case.

The lack of *rho* expression in the ligament cells precludes the possibility that Rho function is required within

these cells for the cleavage and subsequent secretion of an EGFR ligand. However, *rho* is a pleiotropic gene (for a review, see Shilo, 2003) and it is known to affect developmental processes that may affect the development of LA cells indirectly. For example, Rho is known to be required for the recruitment of ch precursors (Okabe and Okano, 1997; zur Lage et al., 1997) and for the establishment of cell identities in the epidermis (O'Keefe et al., 1997). More recently, it has been shown that EGFR signaling is important for late differentiation during peripheral neuron and glia development (Sepp and Auld, 2003). The lch5 organs of *rho* mutant embryos not only lack two ch organs but also appear to have abnormal morphology. The ligament cells show a reduced level of α85E-tub staining as compared to wild-type embryos (Figure 5E), and occasionally the CA cells are not properly stretched or are missing altogether (data not shown). Thus, it is possible that in the absence of *rho* the terminal differentiation of lch5 organs is perturbed, and one of the consequences is the inability of these organs to recruit LA cells.

Another possibility is that Spi and Rho are important for generating a basal activation of the EGFR in ectodermal cells (competence group) through a diffusion of Spi from other neighboring cells and that Vein activity is added to this basal level to finalize the differentiation of LA cells. To establish whether Rho is required in early stages of LA cell development in ectodermal cells, we tested the ability of sSpi expressed in ligament cells to induce the differentiation of supernumerary LA cells in a *rho* mutant background. As shown in Figure 5F, the expression of sSpi in the ligament cells under the regulation of *repo-Gal4* resulted in the formation of increased numbers of normal-looking LA cells, despite the lack of *rho* function in the ectoderm. This observation suggests that Rho and Spi are not essential for early specification of competent cells in the ectoderm. Rather, they may be required within the lch5 lineage.

Concluding Remarks

Proprioceptive organs in many organisms must be stably anchored at both their edges in order to sense relative displacement of movable body parts. The mechanisms that are responsible for the accurate matching between the proprioceptors and their attachment sites have not been characterized yet. In *Drosophila*, the pentascolopidial ch organs are stretched between two attachment sites in the larval body wall. At one edge of the organ, the cap cells attach to the cuticle through lineage-related attachment cells. At the other edge of the organ, we now show that the ligament cells induce the differentiation of highly similar epidermal attachment cells through an EGFR-mediated mechanism. ch organs are found in insects and crustaceans but not in other arthropod classes or in vertebrates. However, despite the great difference in structure, an intriguing parallelism between the signaling cascades that control ch organ development in *Drosophila* and muscle spindle development in the mouse emerges. In both cases, EGFR signaling is activated nonautonomously to induce the expression of an EGR-like transcription factor that is essential for the differentiation of proprioceptive accessory cells.

Experimental Procedures

Fly Strains

Fly strains carrying the following mutant chromosomes were used in this study (described in FlyBase [1999] unless indicated otherwise): *spi*^{OE92} (synonym *spi*^{3}), *gcm*$^{N7-4}$, *repo*^{3702}, hth$^{64-1}$, *sr*^{155}, *abd-A*$^{iab-D24}$, *ut*^{H599}, *rho*$^{\Delta 38}$, vn^{P1749} (described in Yarnitzky et al., 1997), *Df(3R)DG4, and Df(3L)v65c*. The following Gal4 drivers and UAS strains were used: *ato-Gal4* (Hassan et al., 2000), *69B-Gal4* (Brand and Perrimon, 1993), *UAS-GFP;repo-Gal4/TM6* (Sepp et al., 2001; kindly provided by U. Gaul), *UAS-rpr* (Aplin and Kaufman, 1997), *UAS-CD8-GFP;sr-Gal4/TM6* (Subramanian et al., 2003; obtained from G. Morata), *UAS-En-Hth*$^{1-430}$ (Inbal et al., 2001), *UAS-DN-DER* (O'Keefe et al., 1997; kindly provided by B. Shilo), *UAS-sSpi* (Schweitzer et al., 1995; kindly provided by B. Shilo), *UAS-vein* (Yarnitzky et al., 1997). Two *lacZ* reporter strains were used in this study: *rho-lacZ* (*X81*; Freeman et al., 1992) and the enhancer trap strain *sr*^{P1618} (synonym *sr*^{03999}) described in Frommer et al., 1996.

Immunohistochemistry and In Situ Hybridization

Staining of whole-mount embryos was performed using standard techniques (Patel, 1994) with minor modifications. The following primary antibodies were used: rabbit anti-α85E-tub (1/50; Matthews et al., 1990), guinea pig anti-Sr (1/300; Becker et al., 1997), rat anti-Dei (1/200; Yarnitzky et al., 1997), mouse and rabbit anti-β-Gal (1/1000; Promega and Cappel, respectively), mouse anti-βPS Integrin (ascitic fluid 1/100), rabbit anti-Myosin heavy chain (obtained from P. Fisher), mouse anti-Repo (1/10; 8D12, Developmental Studies Hybridoma Bank), mouse anti-Fas3 (1/5; 7G10, Developmental Studies Hybridoma Bank), rabbit anti-Rho (1/1000; Sturtevant et al., 1996), mouse anti-dp-ERK (1/50; Sigma, Saint Louis, Missouri). Secondary antibodies for fluorescent staining were Cy3, FITC, or Cy5-conjugated anti-mouse/rabbit/rat/guinea pig (Jackson). The secondary antibody for nonfluorescent staining was biotinylated anti-rabbit detected with Vecta-Stain Elite ABC-HRP kit (Vector Laboratories).

Whole-mount in situ hybridization using Digoxigenin-labeled probes for *rho*, *β1-tub*, and *vein* was carried out as described in Tautz and Pfeifle (1989) with minor modifications. Stained embryos were viewed using bright-field and confocal microscopy (Zeiss Axioskop and Radiance 2000; BioRad).

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